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DATE: Wednesday, December 04, 2002

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L11	l9 and L10	249	L11
L10	embryo$	18657	L10
L9	l3 and L8	415	L9
L8	agrobacteri$	4418	L8
L7	l5 and l3	970	L7
L6	l1 and L5	183	L6
L5	(transform$ or transgen$)	296737	L5
L4	onion$	6044	L4
L3	onion	5895	L3
L2	(transform$ or transgen$) and L1	183	L2
L1	allium	1405	L1

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Georgia Helmer PhD
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mailbox 9312

L7 ANSWER 1 OF 14 CAPLUS COPYRIGHT 2002 ACS
AN 2002:142892 CAPLUS
DN 136:180165
TI Process for inducing direct somatic embryogenesis and secondary embryogenesis in monocotyledonous plant cells, and rapidly regenerating fertile plants
IN Eudes, Francois Andre Germain; Laroche, Andre J.; Acharya, Surya Narayan
PA Her Majesty the Queen in Right of Canada as Represented by the Minister of Agriculture and Agri-Food, Can.
SO PCT Int. Appl., 70 pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 2


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   PATENT NO.      KIND  DATE        APPLICATION NO.  DATE
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PI WO 2002014520   A2    20020221    WO 2001-CA1165   20010817
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   AU 2001091535    A5    20020225    AU 2001-91535    20010817
PRAI US 2000-641243  A    20000817
   WO 2001-CA1165    W    20010817
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L7 ANSWER 2 OF 14 CAPLUS COPYRIGHT 2002 ACS
AN 2002:123175 CAPLUS

DN 136:178939
TI Gossypium hirsutum tissue-specific promoters and their use
IN Allen, Randy D.; Song, Ping
PA Texas Tech University, USA
SO PCT Int. Appl., 50 pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 1

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PI  WO 2002012450   A1   20020214    WO 2001-US24846  20010807
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           BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG
    AU 2001083190   A5   20020218    AU 2001-83190    20010807
PRAI US 2000-223496P  P  20000807
    WO 2001-US24846   W  20010807
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RE.CNT 3 THERE ARE 3 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L7 ANSWER 3 OF 14 CAPLUS COPYRIGHT 2002 ACS
AN 2002:850253 CAPLUS
TI Process for inducing direct somatic embryogenesis and secondary embryogenesis in monocotyledonous plant cells, and rapidly regenerating fertile plants
IN Eudes, Francois Andre Germain; Laroche, Andre J.; Acharya, Surya Narayan
PA Can.
SO U.S. Pat. Appl. Publ., 31 pp., Cont.-in-part of U. S. Ser. No. 641,243.
CODEN: USXXCO
DT Patent
LA English
FAN.CNT 2

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PI  US 2002164798   A1   20021107    US 2001-929831   20010814
PRAI US 2000-641243  A2  20000817
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L7 ANSWER 5 OF 14 CAPLUS COPYRIGHT 2002 ACS
AN 2000:790647 CAPLUS
DN 133:345572
TI Method for producing transgenic plants resistant to glyphosate herbicides
IN Hawkes, Timothy Robert; Warner, Simon Anthony James; Andrews, Christopher John; Bachoo, Satvinder; Pickerill, Andrew Paul
PA Zeneca Limited, UK
SO PCT Int. Appl., 87 pp.
CODEN: PIXXD2
DT Patent

LA English
FAN.CNT 1

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   PATENT NO.      KIND  DATE          APPLICATION NO.  DATE
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PI  WO 2000066748   A1   20001109      WO 2000-GB1573   20000420
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    EP 1173582      A1   20020123      EP 2000-920929   20000420
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    BR 2000010087   A    20020611      BR 2000-10087    20000420
PRAI GB 1999-17834  A    19990429
    GB 1999-30213   A    19990429
    GB 1999-9968    A    19990429
    GB 1999-17839   A    19990729
    GB 1999-17840   A    19990729
    GB 1999-17846   A    19990729
    GB 1999-17847   A    19990729
    GB 1999-30200   A    19991221
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    GB 1999-30207   A    19991221
    GB 1999-30209   A    19991221
    WO 2000-GB1573  W    20000420
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RE.CNT 10 THERE ARE 10 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L7 ANSWER 6 OF 14 CAPLUS COPYRIGHT 2002 ACS
AN 2000:790646 CAPLUS
DN 133:345571
TI Method for producing transgenic plants resistant to glyphosate herbicides
IN Hawkes, Timothy Robert; Warner, Simon Anthony James; Andrews, Christopher John; Bachoo, Satvinder; Pickerill, Andrew Paul
PA Zeneca Limited, UK
SO PCT Int. Appl., 98 pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 2

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PI  WO 2000066747   A1   20001109      WO 2000-GB1572   20000420
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BR 2000010069 A 20020122 BR 2000-10069 20000420
EP 1173581 A1 20020123 EP 2000-920928 20000420
R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT,
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PRAI GB 1999-17835 A 19990429
GB 1999-9967 A 19990429
GB 1999-9969 A 19990429
GB 1999-9972 A 19990429
GB 1999-9981 A 19990429
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GB 1999-30210 A 19991221
GB 1999-30212 A 19991221
WO 2000-GB1572 W 20000420
RE.CNT 10 THERE ARE 10 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L7 ANSWER 7 OF 14 CAPLUS COPYRIGHT 2002 ACS
AN 2000:790645 CAPLUS
DN 133:345570
TI Method for producing transgenic plants resistant to glyphosate
herbicides
IN Hawkes, Timothy Robert; Warner, Simon Anthony James; Andrews, Christopher
John; Bachoo, Satvinder; Pickerill, Andrew Paul
PA Zeneca Limited, UK
SO PCT Int. Appl., 85 pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 2
PATENT NO. KIND DATE APPLICATION NO. DATE
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PI WO 2000066746 A1 20001109 WO 2000-GB1559 20000420
W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR,
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EP 1173580 A1 20020123 EP 2000-920919 20000420
R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT,
IE, SI, LT, LV, FI, RO
BR 2000010169 A 20020205 BR 2000-10169 20000420
PRAI GB 1999-9971 A 19990429
GB 1999-9972 A 19990429
GB 1999-17837 A 19990729
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GB 1999-30190 A 19991221
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GB 1999-30214 A 19991221

GB 1999-30216 A 19991221
WO 2000-GB1559 W 20000420
RE.CNT 2 THERE ARE 2 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L7 ANSWER 8 OF 14 CAPLUS COPYRIGHT 2002 ACS
AN 2000:790242 CAPLUS
DN 133:330528
TI Transformation of Allium sp. with
agrobacterium using embryogenic callus cultures
IN Reynolds, John
PA Seminis Vegetable Seeds, Inc., USA
SO PCT Int. Appl., 22 pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 1

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI WO 2000065903	A1	20001109	WO 2000-US12463	20000505

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CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG

EP 1180927	A1	20020227	EP 2000-932149	20000505

R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT,
IE, SI, LT, LV, FI, RO
PRAI US 1999-132617P P 19990505
WO 2000-US12463 W 20000505
RE.CNT 3 THERE ARE 3 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L7
FORMAT

L7 ANSWER 12 OF 14 CAPLUS COPYRIGHT 2002 ACS
AN 1992:505726 CAPLUS
DN 117:105726
TI Plant transformation by microparticle bombardment with
Agrobacterium adsorbed to the particles
IN Bidney, Dennis
PA Pioneer Hi-Bred International, Inc., USA
SO Eur. Pat. Appl., 11 pp.
CODEN: EPXXDW
DT Patent
LA English
FAN.CNT 1

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI EP 486234	A2	19920520	EP 1991-310375	19911111

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   EP 486234       A3   19920715
   EP 486234       B1   19950719
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   CA 2053474       AA   19920515     CA 1991-2053474  19911015
   AU 9187714       A1   19920521     AU 1991-87714    19911108
   AU 645857       B2   19940127
   ES 2077182       T3   19951116     ES 1991-310375   19911111
   HU 60782       A2   19921028     HU 1991-3555     19911113
   JP 05308961      A2   19931122     JP 1991-299110   19911114
PRAI US 1990-614403        19901114
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L7 ANSWER 13 OF 14 CAPLUS COPYRIGHT 2002 ACS

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AN  1992:505725  CAPLUS
DN  117:105725
TI  Plant transformation with Agrobacterium using
    microparticle bombardment
IN  Bidney, Dennis
PA  Pioneer Hi-Bred International, Inc., USA
SO  Eur. Pat. Appl., 12 pp.
    CODEN: EPXXDW
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LA  English
FAN.CNT 1
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PI  EP 486233       A2   19920520     EP 1991-310374   19911111
    EP 486233       A3   19920715
    EP 486233       B1   19950719
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    CA 2053473      AA   19920515     CA 1991-2053473  19911015
    AU 9187713      A1   19920521     AU 1991-87713    19911108
    AU 643846       B2   19931125
    ES 2077181      T3   19951116     ES 1991-310374   19911111
    HU 60783        A2   19921028     HU 1991-3556     19911113
    JP 05308960     A2   19931122     JP 1991-299109   19911114
PRAI US 1990-614402        19901114
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L7 ANSWER 14 OF 14 CAPLUS COPYRIGHT 2002 ACS

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AN  1990:2029  CAPLUS
DN  112:2029
TI  Inducible virus resistance in plants
IN  Hohn, Thomas; Bonneville, Jean Marc; Fuetterer, Johannes; Gordon, Karl;
    Sanfacon, Helene
PA  Ciba-Geigy A.-G., Switz.
SO  Eur. Pat. Appl., 24 pp.
    CODEN: EPXXDW
DT  Patent
LA  German
FAN.CNT 1
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PI  EP 298918       A2   19890111     EP 1988-810452   19880701
    EP 298918       A3   19901219
    EP 298918       B1   20010905
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    AT 205253       E    20010915     AT 1988-810452   19880701
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ES 2165345	T3	20020316	ES 1988-810452	19880701
DD 294501	A5	19911002	DD 1988-317674	19880707
DK 8803828	A	19890111	DK 1988-3828	19880708
AU 8818848	A1	19890112	AU 1988-18848	19880708
AU 620039	B2	19920213		
HU 47321	A2	19890228	HU 1988-3615	19880708
HU 207534	B	19930428		
ZA 8804917	A	19890329	ZA 1988-4917	19880708
JP 01037294	A2	19890207	JP 1988-172516	19880711

PRAI CH 1987-2645 A 19870710

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A1

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(30) Priority Data:
60/132,617 5 May 1999 (05.05.99) US

(71) Applicant (*for all designated States except US*): SEMINIS VEGETABLE SEEDS, INC. [US/US]; 1905 Lirio Avenue, Saticoy, CA 93004 (US).

(72) Inventor; and
(75) Inventor/Applicant (*for US only*): REYNOLDS, John [US/US]; 600 Schmeiser Avenue, Davis, CA 95616 (US).

(74) Agents: MUELLER, Lisa, V. et al.; Rockey, Milnamow & Katz, Ltd., Suite 4700, Two Prudential Plaza, 180 North Stetson Avenue, Chicago, IL 60601 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: TRANSFORMATION OF *ALLIUM SP.* WITH *AGROBACTERIUM* USING EMBRYOGENIC CALLUS CULTURES

(57) Abstract

The present invention relates to a method for transforming *Allium* species with a heterologous gene using *Agrobacterium*.

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Transformati n of *Allium sp.* with Agrobacterium Using Embryogenic Callus Cultures

Technical Field of the Invention

The present invention relates to a method for transforming *Allium* species with a heterologous gene using *Agrobacterium*.

Background of the Invention

Transformation in onion has eluded the scientific community. Initial work on the crop centered around use of biolistics as a means of transforming vegetable monocots (Eady, C.C., Weld, R.J. & Lister, C.E. Transformation of onion, *Allium cepa* L., *Proc. Nat. Onion Research Conference*, Sacramento, CA. USA, Dec. 10-12, 1998). No convincing reports were published showing success using this approach. Recent success was reported in transformation of rice, wheat and corn, using *Agrobacterium* based approaches (U.S. Patent 5,591,616). These reports lead to use of *Agrobacterium* for transformation in monocot vegetables. Recently, Eady (Eady, C.C., Weld, R.J. & Lister, C.E. Transformation of onion, *Allium cepa* L, *Proc. Nat. Onion Research Conference*, Sacramento, CA. USA, Dec. 10-12, 1998) at Crop and Food, NZ, reported on successful transformation of onion using *Agrobacterium* with a kanamycin selectable marker and a Green Florescent Protein (GFP) scoreable marker.

Summary of the Invention

In one embodiment, the present invention relates to a method for transforming an *Allium* species, such as *Allium cepa* or *Allium fistulosum*, with a heterologous gene. Specifically, the method involves contacting embryogenic callus material from an *Allium* species with a bacterium belong to the genus *Agrobacterium* which contains a heterologous gene. The embryogenic callus material is preferably derived from immature embryos or flower buds from an *Allium* species. Preferably, the *Agrobacterium* is *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens* and contains a Ti or Ri plasmid. The heterologous gene can be the EPSPS or modified EPSPS gene.

In another embodiment, the present invention further relates to a method for transforming an *Allium* species with a heterologous gene. The first step of the method involves culturing immature embryos or flower buds from an *Allium species* such as *Allium cepa* or *Allium fistulosum* on an initiation medium for a period of from about 2 to about 6 months until embryogenic callus material forms on the embryos or flower buds. Preferably, the immature embryo or flower buds are cultured on the initiation medium in the dark and at a temperature of from about 25°C to about 30°C. The next step of the method involves transferring the embryogenic callus material to a coculture medium and contacting the embryogenic callus material with a suspension of *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens* containing a heterologous gene. The next step involves incubating the embryogenic callus with *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens* for a period of from about 2 to about 4 days. The next step involves removing the *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens* from the transformed embryogenic callus material. The final step involves regenerating the transformed embroygenic callus material into transformed *Allium* plants containing the heterologous gene.

Finally, the present invention relates to an *Allium* species transformed by either of the hereinbefore described methods and progeny thereof.

Detailed Description of the Invention

The present invention relates to a method for transforming onion with a heterologous gene using *Agrobacterium* mediated transformation. Any type of onion can be transformed using the method of the present invention, such as, but not limited to *Allium cepa* and *Allium fistulosum*. As used herein, the term "heterologous" when used to describe a gene refers to a gene that originates from a foreign species, or, if from the same species, is substantially modified from its original form. For example, a promoter operably linked to a heterologous structural gene is from a species different from that from which the structural gene was derived, or, if from the same species, one or both are substantially modified from their original form.

The method of the present invention employs nodular embroygenic callus material. This embryogenic callus material is preferably derived from immature embryos or from flower buds using techniques which are well known in the art. For example, immature embryos can be obtained from up to fourteen (14) day old post-pollinated flowers. Immature flower buds can be obtained from unopened umbels from an onion.

Once the immature embryos or flower buds are obtained, they are placed on a callus initiation medium such as the initiation medium described in Table A as media number one (#1) and kept under appropriate environmental conditions, specifically, in the dark and at a temperature between from about 25°C to about 30°C, to allow the formation of callus. Other initiation media which induce the formation of callus which are well known in the art, can also be used. For example, any salt formulation media, such as but not limited to, Murshige and Skoog (MS) (Murashige T., Skoog F. (1962) *Physilogia Plantarum* 15:473-497), B-5 (Gamborg, O. L., R. A. Miller, and K. Ojima (1968) "Nutrient requirements of suspension cultures of soybean root cells" *Exp. Cell Res.* 50: 148-151), Heller (Heller, R. (1953) "Recherches sur la nutrition minerale des tissus vegetaux cultivers *in vitro*." *Ann. Sci. Natl. Biol. Veg.* 14: 1 223), White (White. P. R. "Nutrient deficiency studies and an improved inorganic nutrient medium for cultivation of excised tomato roots." *Growth* 7: 53 (1943), which contain a high concentration of auxins (such as indole acetic acid (IAA)), 2,4-diclorophenoxy acetic acid, picloram, indole butyric acid (IBA) as well as a carbon source (such as glucose, sucrose, etc) can be used.

After about two (2) to six (6) months, a nodular embryogenic callus forms on the embryos or flowers. The callus is maintained by subculturing every four (4) weeks, keeping the culture in the dark at a temperature between about 25°C to about 30°C. During this period, any tissue which is not nodular embryogenic callus is removed from the culture. Specifically, the removal of brown or smooth textured tissue and of tissue with anthocyanin or sticky exudates faciliates the development of the nodular

embryogenic callus. The nodular embryogenic callus is the material suitable for transformation with *Agrobacterium*.

For regeneration, the nodular embryogenic callus is transferred to a regeneration medium such as the regeneration medium provided for in Table A as media number two (#2) and is placed under Cool White fluorescent light for about fourteen (14) to about eighteen (18) hours per day at a temperature between about 25°C to about 30°C. Other regeneration media which are well known in the art can also be used. For example, any salt formulation medium, such as, but not limited to, Murshige and Skoog (MS), B-5, Heller, White, which contains low levels of cytokinins (such as benzylaminopurine (BA), kinetin, 6-dimethyallyaminopurine (2IP) and a carbon source (such as glucose, sucrose, etc.) can also be used.

Any desired heterologous or target gene can be introduced into *Allium sp.* using the method of the present invention. The heterologous gene used in the method of the present invention encodes for the expression of a protein, such as the 5-enolpyruvyl-3-phosphate synthase enzyme, which conveys resistance to the glyphosate herbicide. The desired heterologous gene to be inserted into onion can be isolated using molecular biology techniques which are well known in the art or can be produced synthetically using molecular biology techniques which are also well known in the art.

As discussed in the previous paragraph, an example of a heterologous gene that can be used in the method of the present invention is a gene which encodes for the 5-enolpyruvyl-3-phosphate synthase enzyme, which conveys resistance to the glyphosate herbicide. As is well known in the art, glyphosate inhibits the shikimic acid pathway which leads to the biosynthesis of aromatic compounds including amino acids, plant hormones and vitamins. Specifically, glyphosate curbs the conversion of phosphoenolpyruvic acid and 3-phosphoshikimic acid to 5-enolpyruvyl-3-phosphoshikimic acid by inhibiting the enzyme 5-enolpyruvyl-3-phosphate synthase (hereinafter referred to as "EPSPS" or "EPSP synthase"). It is well known that glyphosate-tolerant plants can be produced by inserting into the genome of the plant the

capacity to produce a higher level of EPSP synthase in the chloroplast of the cell which enzyme is preferably glyphosate-tolerant.

Many EPSP synthase genes and the use of these genes to transform plants to make plants which are tolerant to glyphosate herbicides are well known in the art. For example, the nucleotide sequence for the mutant *E. coli* EPSP synthase aroA gene was determined by the method of Sanger, et al. (*Proc. Natl. Acad. Sci. USA* 74:5463) and the corresponding amino acid sequence for the encoded EPSP synthase deduced therefrom. U.S. Patent 4,769,061 discloses a mutated aroA gene which expresses 5-enolpyruvyl-3-phosphoshikimate synthase (EC: 2.5.1.19) (ES-3-P synthase) and methods for making plants which express this mutated gene and which exhibited enhanced resistance to glyphosate herbicides. U.S. Patent 4,940,835 discloses a cloning or expression vector comprising a gene which encodes EPSPS polypeptide which, when expressed in a plant cell contains a chloroplast transit peptide which allows the polypeptide, or an enzymatically active portion thereof, to be transported from the cytoplasm of the plant cell into a chloroplast in the plant cell, and confers a substantial degree of glyphosate resistance upon the plant cell and plants regenerated therefrom. U.S. Patent 5,188,642 discloses how to use the vector described in U.S. Patent 4,940,835 to selectively control weeds in a field. U.S. Patents 5,145,783, 4,791,908 and 5,312,910 describe plant genes, methods for producing said genes and vectors containing these genes which encode a glyphosate-tolerant EPSP synthase where the EPSP synthase has an alanine residue substituted for a glycine residue in a conserved sequence found between positions 80 and 120 in the mature wild-type EPSP synthase. U.S. Patents 5,627,061 and 5,310,667 discloses plant genes encoding EPSP synthases and methods for preparing said genes which are prepared by substituting an alanine residue for a glycine residue in a first conserved sequence found between positions 80 and 120, and either an aspartic acid residue or asparagine residue for a glycine residue in a second conserved sequence found between positions 120 and 160 in the mature wild type EPSP synthase. U.S. Patents 5,633,435 and 5,804,425 disclose a modified EPSPS gene from *Agrobacterium sp.* strain CP4. U.S. Patent 5,866,775 discloses plant genes which encode a glyphosate-tolerant EPSP synthase where the EPSP synthase has an alanine residue substituted for a glycine

residue in a conserved sequence found between positions 80 and 120 and a threonine residue for an alanine residue in a second conserved sequence found between positions 170 and 210 in the mature wild-type EPSP synthase. Additional EPSP synthase genes are disclosed in Padgette et al., *Herbicide Resistant Crops*, Lewis Publisher pages 53-85 (1996). Thereupon, any of the hereinbefore described EPSPS genes can be used in the method of the present invention.

The heterologous gene to be expressed in onion can be used to construct an expression cassette which will be introduced into onion. The construction and composition of expression cassettes is well known in the art. Specifically, the elements of the expression cassette are the heterologous gene, a promoter and a termination DNA segment. The heterologous gene is operatively linked to a promoter DNA segments which controls the expression of the heterologous gene. As used herein, the term "operatively linked"includes reference to a functional linkage between a promoter and the heterologous gene, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the heterologous gene. Generally, operably linked means that the nucleic acid sequences being linked are contigous and, where necessary to joint two protein coding regions, contagious and in the same reading frame. This promoter is not repressed by a product of normal onion metabolism, and can be a constitutive promoter such as the CaMV 35S, octopine synthase promoter (P-Ocs) and nopaline synthase promoter (P-Nos) promoters, or organ-enhanced promoters that cause expression in one or more limited organs of the transformed onion.

The final element in the expression cassette is a termination DNA segment that is operatively linked to the 3' end of the heterologous gene. Several termination segments useful in plants are well known in the art and can be used herein. One exemplary segment is the 3' non-translated region of the nopaline synthase gene (Nos-T). Another is the 3'-non-translated region of the pea rbcS-E9 gene.

In addition, the expression cassette can contain a marker gene which confers a selectable phenotype on the onion cells. For example, the marker may encode biocide

resistance, particularly antibiotic resistance, such as resistance to kanamycin, G418, bleomycin, hygromycin, or herbicide resistance, such as resistance to glyphosate or chlorosulforon.

An expression cassette containing the heterologous gene can be introduced into onion using the Ti plasmid of *Agrobacterium tumefaciens* or the Ri plasmid of *Agrobacterium rhizogenes*. The Ti or Ri plasmid is transmitted to plant cells on infection by *Agrobacterium* and is stably integrated into the plant genome. Ti and Ri plasmids contain two regions essential for the production of transformed cells. One of these, named transfer DNA (T-DNA), is transferred to plant nuclei and induces tumor or root formation. The other, termed the virulence (vir) region, is essential for the transfer of the T-DNA but is not itself transferred. The T-DNA will be transferred into a plant cell even if the vir region is on a different plasmid. The transferred DNA region can be increased in size by the insertion of heterologous DNA without its ability to be transferred being affected. Thus, a modified Ti or Ri plasmid, in which the disease-causing genes have been deleted, can be used as a vector for the transfer of the gene constructs of this invention into an appropriate plant cell. Construction of recombinant Ti and Ri plasmids in general follows methods typically used to introduce additional DNA into the more common bacterial vectors, such as pBR322. Additional use can be made of accessory genetic elements sometimes found with the native plasmids and sometimes constructed from foreign sequences. These may include, but are not limited to, "shuttle vectors" and structural genes for antibiotic resistance as a selection factor.

The nodular embryogenic callus material prepared as described above is then contacted with the Ti or Ri plasmid of *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* which contains the expression cassette with the heterologous gene. After the embryogenic callus material is contacted with the *Agrobacterium*, it is then incubated for about two (2) to about four (4) days at a temperature of about 20°C to about 25°C in the dark. After the incubation period, the *Agrobacterium* is removed or disinfected such as by scraping callus tissue into a dish with wash media, such as the wash medium described in Table B, agitating it and then removing the wash medium.

After removal of the *Agrobacterium*, the washed embryogenic callus material is transferred to a selection medium, such as the selection medium described in Table A as media number four (#4). Other selection media, which are well known in the art, such as media containing the antibiotic kanamycin, can also be used. The callus cultures are grown under Cool White fluorescent light for about 14 to about 18 hours per day at a temperature between about 25°C to about 30°C.

After about thirty (30) days, the callus is subcultured onto a second higher selection media, such as the selection medium described in Table A as media number five (#5), for all following transfers. Selection transfers are done every four (4) weeks per subculture.

Any remaining callus which is living and is producing embryos or plants is then transferred to the rooting media in 0.05 mM glyphosate which is described in Table A as media #6 for final regeneration. Other rooting media which are well known in the art can also be used. The regenerating shoots are grown under Cool White fluorescent light for about 14 to about 18 hours per day at a temperature between about 25°C to about 30°C. Regenerated and rooted shoots are then transplanted into pots filled with soil under high light intensity, such as 1000 foot candles, and at near 100% relative humidity, such as by covering the pots with plastic.

The shoots are allowed to continue to grow and develop into transformed *Allium* plants which contain the heterologous gene. Transformed plants containing the heterologous gene described herein can be identified using techniques known in the art such as Northern or Southern Blotting or polymerase chain reaction.

By way of example and not of limitation, examples of the present invention will now be given.

Example 1: Materials and Methods

a. Callus initiati n- Immature embryos from onion, specifically, *Allium cepa* or *Allium fistulosum*, were isolated under a dissecting microscope from approximately 14 day post pollinated flowers. Flower heads can be shipped overnight from various breeding stations around the US, refrigerated and used as explant source for a period of about one (1) to about two (2) weeks. Individual flower buds were removed from the umbel and placed in a 15ml screw cap centrifuge tube. Full strength Clorox plus 0.5% Tween 20 were added to the tube and mixed every 2-3 minutes for 15 minutes. Clorox was removed and buds were washed 4 times with sterile Reverse Osmosis (RO) water. Embryos were isolated by placing the bud on a sterile Petri dish under a 40x dissecting microscope with the flower base facing up. Using a #11 scalpel, the base of the flower was cut to the point of just removing the bottom of the pollinated seed. The seed coat is black and the endosperm is milky to doughy in consistency. The embryos can be squeezed out of the incision on the bottom of the seed with forceps pressure on the top third of the flower bud. However, this procedure may not be successful with older flowers where the endosperm is harder and the embryo is larger. Under these conditions, the seed is extracted from the flower bud for individual embryo excision. These embryos are excised by slicing down the seed coat on the side where the embryo is located. The embryo is extracted from the seed through the incision. Embryos are lifted from the plate on the scapel tip and placed on callus initiation medium (described in Table A as medium #1). Embryos range in size from 1-5 mm.

Plates 60x20mm containing 40ml media can hold up to 25 embryos. A nodular callus forms on the embryo after about 2 to about 4 months. Callus is maintained by subculture for about 3 to about 4 weeks on callus medium #1 shown in Table A. Callus tissue is grown at about 28°C in the dark. Selection of nodular embryogenic tissue is important at each subculture. Removal of brown or smooth consistency tissue, tissue with anthocyanin or sticky exudates promotes development of embryogenic callus.

b. Callus regeneration- Nodular selected tissue is transferred to 60x20mm plates containing 40ml of regeneration medium (described in Table A as medium #2). Cultures are placed under 100 foot candles of Cool White fluorescent light for 16 hours per day at a temperature of about 28° C. Tissue is subcultured at about 3 to about 4 weeks, with embryo regeneration seen at 6-8 weeks.

c. Callus transformation- *Agrobacterium tumefaciens* cultures are initiated from streaked plates of freezer stock. Two loops of plate stock or 100ul of freezer stock are placed in 5ml YEP medium (described in Table B) containing appropriate antibiotics in a 25x150mm tube and placed on a roller drum in room light. Overnight cultures are subcultured by adding 5ml of the overnight culture to 50ml of AB medium (described in Table B) with antibiotics and grown in the dark overnight at 28°C on a gyratory shaker. The next day identified regenerable callus is placed on glass filter paper over co-culture medium (described in Table A as medium #3). Callus tissue is placed on the filter paper at a moderate density. Only nodular tissue is selected for transformation. Overnight *Agrobacterium* cultures are adjusted to an optical density (OD) of from about 0.1-0.4, preferably 0.4, at 660nm with dilution medium (Table B). Diluted cultures are drawn into a plastic sterile transfer pipette. Callus tissue is dabbed with the end of the pipette so a small amount of solution covers the callus tissue. Each callus piece in the plate is touched. The plates are sealed with Parafilm, placed in a black plastic box and incubated at 23°C for 3 days. On day three, *Agrobacterium* is removed by scraping tissue into a 60x20mm plate containing 10ml of wash medium as described in Table B. Tissue is agitated with a transfer pipette followed by removal of the wash. Tissue is scraped into 40ml selection media (described in Table A as medium #4) in a 60x20mm Petri dish and sealed with Parafilm. Cultures are grown under 100 foot candles Cool White florescent light for 16hr/day. After one month, callus is subcultured into a second selection media (described in Table A as medium #5) for 2 transfers and back to selection media #4 (described in Table A) for 1 transfer. Any living callus is transferred to medium #2 (described in Table A) without selection for final regeneration. Regenerating embryos are placed on 50ml rooting medium (described in Table A as medium #6) in Magenta containers and grown under similar light conditions.

Example 2: Specific Experiments

Experiment 212. Callus material used in this experiment was initiated from immature embryos from proprietary *Allium cepa* breeding material owned by Seminis Vegetable Seeds, Inc. Pollinated flowers were sent from Las Cruses, New Mexico to Woodland, California and immature embryos were isolated, using the procedures described in Example 1a from 11 proprietary *Allium cepa* lines. Callus, recently subcultured for seventeen days, from the proprietary *Allium cepa* lines 197,195, 193 and 248 were cocultured on medium #3 (described in Table A) for three days with disarmed Agrobacterium strain ABI containing Monsanto CP4 construct pMON10147 (Monsanto Company, St. Louis, Missouri). The construct pMON10147 contains the enhanced 35S promoter from figwort mosaic virus (which is disclosed in U.S. Patent 5,633,435, hereby incorporated by reference), the leader sequence from the Petunia heat shock protein 70 (HPS70) (disclosed in Winter J., et al., *Mol. Genet.* 211:315-319 (1988), hereby incorporated by reference), the chloroplast transit peptide sequence (CTP2) of the 5-enolpyruvylshikimate-3-phosphate synthase gene (EPSPS) from *Arabidopsis thaliana* which is also disclosed in U.S. Patent 5,633,435, the "modified" EPSPS gene from *Agrobacterium sp.*strain CP4 which is disclosed in U.S. Patent 5,633,435 and the 3' region from the small subunit of ribulose-1,5-bisphosphate gene from *Pisum sativum* (E9) which is also disclosed in Coruzzi, G., et al., *EMBO J.* 3:1671 (1984) and Morelli, G., et al., *Nature*, 315:200-204 (1985), hereby incorporated by reference.

The construct also contains the 35S promoter from cauliflower mosaic virus (CaMV), the chloroplast transit peptide sequence of the small subunit 1a (SSU1a) gene from *Arabidopsis thaliana (*disclosed in Timko M P., Herdies L., Alameida E., Cashmore A R., Leemans J. & Krebbers E. (1988) Genetic engineering of nuclear-encoding components of the photosynthetic apparatus of Arabodopsis. *In* The impact of chemistry on biotechnology – a multidisiplinary discussion- (Phillips M., Schoemaker S.P., Middlekauff D. & Ottenbrite R.M. eds) ACS Books, Washington DC, pp. 279-295), herein incorporated by reference), the modified glyphosate oxidoreductase gene

(GOXsyn) from *Achromobacter sp.* (which is also disclosed in U.S. Patent 5,633,435) and the 3' region of the nopaline synthase gene (nos) from *Agrobacterium tumafaciens* T-DNA.

a. The binary ABI strain contains the disarmed (lacking the T-DNA phytohormones) pTiC58 plasmid pMP9ORK (Koncz, C. and Schell, J., 1986. "The Promoter of TL-DNA Gene 5 Controls the Tissue-Specific Expression of Chimeric Genes Carried by a Novel Type of Agrobacterium Binary Vector," *Mol. Gen. Genet.* 204: 383-396.), in a chloramphenicol resistant derivative of the Agrobacterium tumefaciens strain A208. The pMP9ORK Ti plasmid was engineered to provide the gene functions required for autonomous replication of the plasmid vector after conjugation into the ABI strain. It also provides the vir functions needed for transfer of the T-DNA into the plant cell.

Callus was transferred, after washing, to callus medium #2 (described in Table A) without selection and grown in the dark. Callus was subcultured after 4 weeks on regeneration medium #4 (described in Table A) with 0.1mM glyphosate and moved to the light. Callus was cultured for 3 additional months, with monthly transfers on 0.1mM glyphosate selection (on medium #4 described in Table A) totaling 4 months. Callus line 248 initially established on Gelrite solidified medium (which is medium#1 described in Table A) produced 2 callus lines after glyphosate selection. These lines were subcultured on regeneration medium #2 (described in Table A) without selection. After 2 months , plants were placed on rooting medium #6 (described in Table A).

b. Experiment 268. This experiment employed additional immature embryos obtained from the proprietary line described above in Example 2a. These embryos underwent callus transformation as described above in Example 1c. Moreover, additional callus material used in this experiment was initiated from immature onion flower tissue which originated from proprietary onion line of Seminis Vegetable Seeds, Inc. which is derived from a cross of *Allium fistulosum* x *Allium cepa*. Amphidiploid plant materials of the original *Allium fistulosum* x *Allium cepa* cross (after colchicine-induced chromosome

doubling) was released by Gil McCollum at the U.S.D.A, Beltsville (Notice of Release of Onion Germplasm f-c 8434, 8492, 8497 and 8615, USDA, ARS, Feb. 2, 1988).

To initiate callus from flowers, unopened umbels were cut and sterilized in 20% Clorox for 5 minutes then rinsed with sterile water. Whole flower buds were excised from the umbels and cultured 20 per plate on callus initiation medium #1 (described in Table A). Callus was maintained with monthly subcultures. Eleven flower callus lines were tested for regeneration and found not to regenerate at the frequency of immature embryo derived material. Flower callus line 290011, identified as a regenerating line, was used in experiment 268 along with 16 other embryo derived or flower derived callus lines. Callus was 15 days into its most recent subculture. Callus was cocultured for 3 days with ABI bacteria containing the Monsanto CP4 construct pMON45312 (Monsanto Company, St. Louis, Missouri). Construct pMON45312 contains the enhanced 35S promoter from figwort mosaic virus (FMV) (which is disclosed in U.S. Patent 5,633,435, hereby incorporated by reference), the chloroplast transit peptide sequence (CTP2) of the 5-enolpyruvylshikimate-3-phosphate synthase gene (EPSPS) from *Arabidopsis thaliana* (which is also disclosed in U.S. Patent 5,633,435) , the leader sequence from the soybean heat shock protein (native 17.9) (disclosed in Arfchke, E., et al., *J. Molec. Bio.* 199:549-557 (1988), herein incorporated by reference), the "modified" EPSPS gene from *Agrobacterium* sp. strain CP4 (which is also disclosed in U.S. Patent 5,633,435) , and the 3' region from the small subunit of ribulose-1,5-bisphosphate gene from *Pisum sativum* (E9) which is also disclosed in Coruzzi, G., et al., *EMBO J.* 3:1671 (1984) and Morelli, G., et al., *Nature*, 315:200-204 (1985), hereby incorporated by reference.

The ABI binary *Agrobacterium* strain pTiC58 contains the disarmed (i.e. lacking the T-DNA phytohormone genes) plasmid pMP9ORK (Koncz, C. and Schell, J., 1986. "The Promoter of TL-DNA Gene 5 Controls the Tissue-Specific Expression of Chimeric Genes Carried by a Novel Type of Agrobacterium Binary Vector," *Mol. Gen. Genet.* 204: 383-396), in a chloramphenicol resistant derivative of the *Agrobacterium tumefaciens* strain A208. The pMP9ORK Ti plasmid was engineered to provide the gene functions

required for autonomous replication of the plasmid vector after conjugation into the ABI strain.

Tissue was inducted after washing on regeneration medium #4 (described in Table A) containing 0.05mM glyphosate and grown in the light. After one month, callus was moved to regeneration media #5 (described in Table A) containing 0.1mM glyphosate for 2 transfers. Callus was transferred back to 0.05mM glyphosate regeneration media #4 (described in Table A) for one month. Selected green callus areas were placed on regeneration media #2 (described in Table A) without selection for 2 months. Developing embryos were transferred to elongation rooting medium #6.

Example 3: Discussion

The choice of tissue for transformation in onion or any plant culture system is critical for successful production of transgenic plants. Experiment 212 used immature embryo derived callus of a proprietary *Allium cepa* line. Two selected callus lines which were transformed were regenerated from this experiment aided by the use of a regenerating embryogenic callus line as the initial tissue source.

Immature flowers may also be used as a callus source. Experiment 268 discloses using onion flowers as callus source, however, the initial regeneration screen showed poor regeneration in flower derived callus. The regenerating flower tissue used in Experiment 268 came from a proprietary line which was a *Allium fistulosum* x *Allium cepa* cross that was doubled to become tetraploid. It appeared to be very vigorous in culture and was one of the only flower derived lines that regenerated.

Experiments 212 varies from 268 by selection procedure although both produced transgenic callus lines. Experiment 212 callus was placed on a callus medium without selection and grown the dark. After 1 month, callus was moved to the light and selected on 0.1mM glyphosate for 4 months. Experiment 268 was directly selected on 0.05mM glyphosate on a regenerating medium in the light followed by 2 months selection on

0.1mM glyphosate and a final selection on 0.05mm glyphosate. Experiment 268 produced more lines, however, different genotypes were used.

Delay of selection is used in soybean glyphosate transformation and should be tested further in the onion procedure, however, selection immediately after coculture, as in experiment 268, produced transgenic lines. The reduction of glyphosate selection was done in experiment 268 due to the fact that glyphosate accumulates in tissue and may overwhelm any engineered plant resistance. This is also why regeneration is done without glyphosate selective pressure.

The present invention is illustrated by way of the foregoing description and examples. The foregoing description is intended as a non-limiting illustration, since many variations will become apparent to those skilled in the art in view thereof. It is intended that all such variations within the scope and spirit of the appended claims be embraced thereby.

Changes can be made to the composition, operation and arrangement of the method of the present invention described herein without departing from the concept and scope of the invention as defined in the following claims.

TABLE A

Onion Media	Callus #1	Regeneration #2	Coculture #3	Selection #4	Selection #5	Rooting #6
MS Salt	4.3 g/l	4.3 g/l	4.3 g/l	4.3 g/l	4.3 g/l	4.3 g/l
B-5 Vitamins	1ml/l	1 ml/l	1 ml/l	1 ml/l	1 ml/l	1 ml/l
Sucrose	30g/l	30 g/l	30g/l	30 g/l	30 g/l	30 g/l
Picloram	1 mg/l					
BA	0.9 mg/l	1 mg/l	1 mg/l	1 mg/l	1 mg/l	
Proline		2.5 g/l	2.5 g/l	2.5 g/l	2.5 g/l	
NaH_2PO_4						170 mg/l
Casein						1 g/l
Kinetin						1 mg/l
Acetosyringone			40 mg/l			
Carbenicillin				500 mg/l	500 mg/l	
Cefotaxime				400 mg/l	400 mg/l	
Glyphosate				0.05mM	0.1mM	0.05mM
Agar // or	7 g/l	7 g/l	7 g/l	7 g/l	7 g/l	6.2 g/l
Phytogel	2.5 g/l					
pH	5.7	5.7	5.7	5.7	5.7	5.8

Table B

YEP Medium
Peptone- 10 g/l
Yeast extract- 10 g/l
NaCl- 5 g/l

AB Medium
Buffer : 20X Final Volume= 500ml
K_2HPO_4. 3H2O- 39.33 g
NaH_2PO_4.H2O- 11.5 g
Filter Sterilize and refrigerate

Salts: 20X Final Volume= 500ml

NH_4Cl-	10g
$MgSO_4.7H_2O$-	12.5g
KCl-	1.5g
$CaCl_2$	0.1g
$FeSO_4$	25mg

Filter Sterilize and refrigerate

Glucose-
50 g/ 500ml

Dilution Medium-
1/10 MSO + 1.0 mg/l BA + 2.5 g/l proline
200uM Acetosyringone
1mM galacturonic acid
20mM MES (2-[N-morpholino]ethanesulfonic acid)
pH 5.4

Wash
MSO (MS medium plus minimal organics)
500ug/l Carbenicillin
400 ug/l Cefotaxime

WHAT IS CLAIMED IS:

1. A method for transforming an *Allium* species with a heterologous gene, the method comprising the step of: contacting embryogenic callus material from an *Allium* species with a bacterium belonging to the genus *Agrobacterium* which contains a heterologous gene.

2. The method of claim 1 wherein the *Allium* species is *Allium cepa* or *Allium fistulosum*.

3. The method of claim 1 wherein the bacterium belonging to the genus *Agrobacterium* is *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens*.

4. The method of claim 1 wherein the bacterium belonging to the genus *Agrobacterium* contains a Ti plasmid or a Ri plasmid.

5. The method of claim 1 wherein the heterologous gene is the EPSPS gene.

6. The method of claim 5 wherein the heterologous gene is a modified EPSPS gene.

7. The method of claim 1 wherein the embryogenic callus material is derived from immature embryos or flower buds from an *Allium* species.

8. An *Allium* species transformed by the method of claim 1 and progeny thereof.

9. A method for transforming an *Allium* species with a heterologous gene, the method comprising the steps of:

a. culturing immature embryos or flower buds from an *Allium* species on an initiation medium for a period of from about 2 to about 6 months until embryogenic callus material forms on the embryos or flower buds;

b. transferring the embryogenic callus material to a coculture medium and contacting the embryogenic callus material with a suspension of *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens* containing a heterologous gene;

c. incubating the embryogenic callus material with the *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens* for a period of from about 2 to about 4 days; and

d. removing the *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens* from the transformed embryogenic callus material.

10. The method of claim 9 wherein the *Allium* species is *Allium cepa* or *Allium fistulosum*.

11. The method of claim 9 wherein the immature embryos or flower buds are cultured on the initiation medium in the dark and at a temperature of from about 25°C to about 30°C.

12. The method of claim 9 wherein the heterologous gene is the EPSPS gene.

13. The method of claim 12 wherein the heterologous gene is a modified EPSPS gene.

14. The method of claim 9 further comprising the step of regenerating the transformed embryogenic callus material into transformed *Allium* plants containing the heterologous gene.

15. An *Allium* species transformed by the method of claim 9 and progeny thereof.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/12463

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A01H 1/00; C07H 21/04; C07K 14/415; C12N 5/04, 5/14, 9/00, 15/00
US CL :435/419, 252.3, 320.1; 530/370; 536/23.2, 23.6; 800/278, 294, 300
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/419, 252.3, 320.1; 530/370; 536/23.2, 23.6; 800/278, 294, 300

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, WEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,424,412 A (BROWN et al.) 13 June 1995, see entire document.	1-15
Y	US 5,767,377 A (NAKAJIMA et al.) 16 June 1998, see entire document.	1-15
Y	EADY et al. Transient expression of uidA constructs in in vitro onion (Allium cepa L.) cultures following particle bombardment and Agrobacterium-mediated DNA delivery. Plant Cell Reports. 1996, Vol. 15, No. 12, pages 958-962, see entire document.	1-15

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
30 JUNE 2000	09 AUG. 2000

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(54) Title: HERBICIDE RESISTANT PLANTS

(57) Abstract

The present invention provides, inter alia, an isolated polynucleotide comprising a region encoding a chloroplast transit peptide and a glyphosate resistant 5-enolpyruvylshikimate phosphate synthase (EPSPS) 3' of the peptide, the said region being under expression control of a plant operable promoter, with the provisos that the said promoter is not heterologous with respect to the said region, and the chloroplast transit peptide is not heterologous with respect to the said synthase.

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Eady t al, Plant Cell Reports 18; 117-121 (1998)

Eady t al, Plant cell Reports, 18, 111-116 (1998)

Eady et al, Plant Cell Reports. 15, 958-962 (1996)

Dommisse, et al Plant Sciencedm 69, 249-257 (1990)

Klein, et al, Nature 327, 70-73. (1987).

Thanks in advance,

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Plant Cell Reports

Transient expression of *uidA* constructs in *in vitro* onion (*Allium cepa* L.) cultures following particle bombardment and *Agrobacterium*-mediated DNA delivery

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Abstract

Particle bombardment and *Agrobacterium*-mediated DNA delivery into immature embryos and microbulbs were used to investigate the expression of the *uidA* gene in *in vitro* onion cultures. Both methods were successful in delivering DNA and subsequent *uidA* expression was observed. Optimal transient β-glucuronidase activity was observed in immature embryos that had been pre-cultured for three days and bombarded at a distance of 3 cm from the stopping plate, under 25 in Hg vacuum, using 900-1300 psi rupture discs. The CaMV35S-*uidA* gene construct gave five fold higher transient β-glucuronidase activity than the *uidA* gene construct regulated by any of four other promoters initially chosen for high experession in monocotyledonous tissues.

Abbreviations: GUS, β-glucuronidase; IE, immature embryo; MUG methylumbelliferyl β-D-glucuronide.

Key words: Transformation, particle bombardment, *Agrobacterium*, *Allium cepa*.

Introduction

Foreign gene transfer to plants is becoming a routine technique for many important crop species. The most commonly used techniques are either *Agrobacterium*-mediated transformation or direct gene transfer by particle bombardment (Fisk and Dandekar 1993), although many other, lesser used, techniques are available (reviewed by Songstad et al. 1995). Despite the availability of these numerous approaches some important crop plants have still proved to be recalcitrant to genetic transformation and plant regeneration. The onion *Allium cepa* and the related vegetables , e.g. garlic (*A. sativum*) and leek (*A. ampeloprasum*), are such recalcitrant crops. These crops have an annual global trade in bulbs worth US$400 million (Brewster 1994). Successful transformation and regeneration of onion has not been achieved although susceptibility to *Agrobacterium* was demonstrated by Dommisse et al. (1990). For a review of onion tissue culture and possible gene transfer systems refer to Eady (1995). Recently Buitveld and Creemers-Molenaar (1994) demonstrated that it is possible to regenerate leeks from protoplasts derived from immature embryo cultures. These examples of gene transfer and totipotency suggest that this important family of vegetables should also be amenable to genetic transformation.

In this paper we use two DNA delivery systems to identify regulatory sequences that drive high levels of *uidA* expression in onion tissues. We optimised the physical parameters governing biolistic transfer of DNA into onion tissues that are capable of regeneration.

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Materials and Methods

Tissue Culture: Mature and immature onion seeds from Canterbury Longkeeper, Sapporo Yellow and Australian Brown cultivars were surface sterilised by immersion in ethanol for 1 minute followed by washing in 30% household bleach (5% NaOCl) for 30 minutes. Seeds were rinsed four times in sterile water prior to processing.

'Microbulbs' were produced by germinating mature seeds on BDS medium (Dunstan and Short 1978) containing 0.2 mg/l of gibberellic acid. After germination the central portion of the seedling, containing the shoot meristem, was excised and placed onto BDSX medium (Shahin and Kanenko 1986) supplemented with 900 mg/l of casein hydrolysate, 2 mg/l benzylaminopurine and 0.75 mg/l picloram (Sigma). Cultures were usually maintained in the dark at 28°C for 2 weeks; any treatments requiring longer culture were maintained by monthly sub-culture. On this medium the base of the cotyledonary sheath, containing the proliferating shoot meristem, swells and resembles a small bulb. These 'microbulbs' were cut transversely just above the widest point and placed, cut surface uppermost, within a 2 cm circle, in the centre of a fresh plate of medium just prior to bombardment.Immature embryos (IEs), 0.5 - 3.5 mm in length, isolated from immature seeds were placed directly onto BDS medium supplemented with 200 mg/l casein hydrolysate and 2.0 mg/l 2,4 - dichlorophenoxyacetic acid. Cultures were maintained on this medium, in the dark at 28°C, by sub-culture every two weeks.

Prior to bombardment approximately 20-40 IEs were placed within a 2 cm circle on fresh medium.

Plasmid DNA: Six different plasmids containing promoter - *uidA* gene constructs were used (Fig.1). Plasmid DNA was isolated using standard phenol/chloroform purification and ethanol precipitation methods (Maniatis et al. 1982). For comparison pART8 DNA was isolated using Wizard^{TM} maxipreps (Promega) according to the manufacturer's instructions. pAHC25 containing the ubiquitin promoter - *uidA* construct (Christensen et al. 1992) was a gift from Professor P. Quail, University of California, USA. pJIT60AoPR1GUSint containing the asparagus promoter *AoPR1* - *uidA* construct (Ozcan et al. 1993) was a gift from Dr. J. Draper, University of Leicester, UK. pAct1-D containing the rice Act1 promoter - *uidA* construct (Zhang et al. 1991) was a gift from Professor R. Wu, Cornell University, USA. pEMU containing the synthetic monocotyledonous promoter - *uidA* (Last et al. 1991) was a gift from Professor W. Peacock, CSIRO, Canberra, Australia. pART8 containing the 1.3Kb CaMV35S promoter region - *uidA* construct was a gift from Dr. A. Gleave (1992), HortResearch, New Zealand. p35SGmtx containing a 1Kb CaMV35S promoter region - *uidA* construct was supplied by Dr. D. Becker (unpublished), Max-Planck-Institut fur Zuchtungsforschung, Germany. This *uidA* gene contained a 189 nucleotide intron isolated from the *st-ls1* gene in potato and was designed to stop GUS activity in bacteria.

Bombardment: Microbulbs and IEs were bombarded using a Dupont biolistic PDS-1000/He particle delivery system. Plasmid DNA (1 mg/ml) was precipitated onto gold particles using the following mixture: 50 ml gold suspension (3 mg gold); 10 ml plasmid DNA; 50 ml 2.5 M calcium chloride and 20 ml 0.1 M spermidine. The mix was vortexed during preparation and for a further 3 minutes. The coated gold was recovered by centrifugation and washing in 0.25 ml of ethanol before being re-suspended in 60 ml of ethanol. 10 ml of the suspension was used per bombardment.

Agrobacterium : *Agrobacterium tumefaciens* strain Agli (Lazo et al. 1991) containing a pGA643 binary vector (An et al. 1988), modified to include a CaMV35S promoter - tobacco mosaic virus untranslated leader (omega fragment) - *uidA* gene construct (a gift from Oi Wah Liew, University of Lincoln, NZ), was grown for 24 hours on luria broth medium (Maniatis et al. 1982) supplemented with 5 mg/l tetracycline, 100 mg/l streptomycin and 100 mM acetosyringone. Microbulbs and IEs were cultured for three weeks and 1 week respectively prior to inoculation. Tissues were immersed in the bacterial suspension for 1 hour and then transferred, without rinsing, to the appropriate media containing 100 mM acetosyringone and incubated in darkness for 5 days. After cocultivation tissues were rinsed thoroughly in sterile water containing 200 mg/l timentin before being placed on fresh medium containing 200mg/l timentin. GUS activity was analysed 5 days after initial infection.

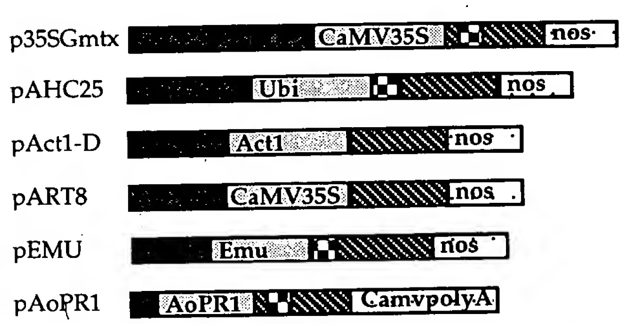

Fig. 1. Plasmids used in bombardment experiments. promoter, intron, *uidA* gene, terminator, remainder of plasmid.

Analysis of transient GUS activity: GUS activity was analysed 2 days after bombardment by histological staining of tissues (Jefferson et al. 1987). Samples were immersed for 5 hours in 0.1M phosphate buffer containing 1mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide; 2.5mM potassium ferricyanide and 2.5mM potassium ferrocyanide (Eady et al. 1994).

The tissue area, size and number of GUS foci was recorded in bombardment experiments using image analysis (Video pro 32 Leading Edge, Pty, Adelaide, Australia) essentially as described by Owens y de Nova and Coles (1994). *Agrobacterium*-mediated transformation events were recorded by observation with a binocular microscope.

Results and Discussion

Image analysis was used to measure the area of tissue bombarded and the number of GUS foci, to give GUS activity per unit tissue area. Analysis of bombarded tissue extracts, using MUG as a substrate, was not performed as this would give data relative to the amount of tissue being bombarded and not just the area. The depth of tissue varied considerably in some samples, and it was concluded that MUG analysis would incorporate significant variation due to tissue size differences. Values recorded as per shot (Becker et al. 1994, Vain et al. 1993) or per embryo (Casas et al. 1993) do not take account of the tissue area. Within each set of data, three control bombardments were performed. One micron gold particles coated with pART8 were delivered at 1100 - 1300 psi into 2 week old Canterbury Longkeeper microbulbs or 2-3 day pre-cultured IEs placed 6 cm from the stopping plate under 25 in Hg vacuum. Results from these bombardments represented 100% activity for microbulbs or IEs; this insured that the relative strength of *uidA* gene expression was accurate.

Table 1 (experiment 1) shows the variation in relative *uidA* expression of bombardments performed by the same operator or by different operators. The greater variation observed between operators was expected as several steps in the bombardment procedure (e.g., sample preparation and microprojectile loading) are difficult to standardise. Subsequent experiments were performed by single operators. As a comparison with other work, control IE bombardments produced on average 17.7 (S.E. of the mean 0.12) foci/mm^2 or approximately 83.6 foci/embryo or 3350 foci/shot; microbulb bombardments produced 6.3 (S.E. of the mean 0.6) foci/mm^2 or approximately 54 foci/microbulb or 874 foci/shot. The number and intensity of blue foci generated in these experiments compares favourably to reports in other species (Casas et al. 1995, Becker et al. 1994) and is similar to those reported by Gallo-Meagher and Irvine (1993) and Vain et al. (1993), using a particle inflow gun. If the frequency of stable integration events in onion is similar to other monocotyledenous species, then these results suggest that the DNA delivery system should not be rate limiting for onion transformation.

Physical parameters affecting bombardment

The physical parameters of bombardment, rupture disc pressure, sample distance, vacuum pressure, gold size, and the presence of a mesh over the sample, are all presented in Table 1 (experiment 2, 3 and 4). The best

Table 1. The effect of physical and tissue parameters on the percentage of GUS foci relative to control bombardments produced in microbulbs.

Exp't	Parameter	% of GUS foci / mm^2 relative to control bombardments (standard error)	
1	**Controls**		
	One operator	100 (9)	
	Two operators	100 (20)	
2	**Sample distance**		
	3 cm	285 (214)*	
	6 cm	100 (33)	
	9 cm	23 (21.4)	
	12 cm	0.4 (0.4)	
3	**Disc rupture pressure**	**6 cm**	**3 cm‡**
	450psi	20 (5)	-
	900psi	87 (17)	204 (16.8)
	1550psi	100 (59)	140 (30)
	1800psi	86 (45)	87** (10)
4	**Vacuum pressure**		
	25in Hg	100 (16)	
	15in Hg	3.9 (0.1)	
	10in Hg	0.6 (0.1)	
	Gold size		
	1.0 micron	100 (16)	
	1.5-3.0 micron	113 (4.6)	
	Sample mesh		
	100 micron mesh	106 (27)	
5	**Culture age before shooting**		
	1 week	136 (19.5)	
	2 week	100 (19.5)	
	4 week	37 (30)	
	8 week	52 (20)	
	10 week	16 (16)	
	Cultivar †		
	Canterbury Longkeeper	136 (19.5)	
	Sapporo Yellow	141 (78)	
	Australian Brown	63 (14)	

Experiment 1 - The effect of using multiple operators to perform bombardments. Experiment 2 - The effect of increasing the distance between the sample and the macroprojectile stopping screen. Experiment 3 - The effect of increasing the disc rupture pressure at 6 cm and 3 cm sample distance. Experiment 4 - The effect of decreasing the sample chamber vacuum pressure, increasing gold particle size, and addition of a protective mesh placed over the sample. Experiment 5- The effect of culture age prior to shooting and cultivar type. * - a large variation arose as shots occasionally missed the target at 3cm. ** - some embryos were displaced by the shock wave. ‡ - average of two sets of data only. † - cultivars were bombarded after 1 week in culture.

results were obtained using a 900 psi rupture disc with the sample 3 cm from the stopping plate and under a vacuum of 25 in Hg. Increasing the size of the gold or placing a mesh over the sample had little effect on the number of GUS foci produced. Plasmid concentration and plasmid isolation method were also investigated. Increasing the plasmid concentration in the gold suspension caused the gold to clump during preparation and led to increased tissue damage after shooting. Plasmid isolated using Wizard™ maxipreps (Promega) produced very few foci when bombarded into onion tissues compared to phenol/chloroform purification and ethanol precipitation methods (data not shown). Onion tissue responded to altering the physical parameters in the same way as other tissues (reviewed by Casas et al. 1995), except that in this study, optimal sample distance was shown to be 3 cm, (usually 6 cm). However, samples placed 3cm were occasionally displaced by the shock wave, and tissue damage was likely to be more serious at this level than at 6 cm.

Effect of tissue type and age on bombardment

The choice of tissue explant is crucial in the development of a transformation system (Casas et al. 1995). Microbulbs and IEs were choosen as both show high frequency regeneration (Eady 1995, Buitveld et al. 1994). In onion IEs regeneration is via embryogenesis, as in other cultured IEs (Vasil et al. 1993). Both tissues were shown to be susceptible to transformation, although bombardment of microbulbs produced 6.3 (S.E. of the mean 0.6) GUS foci per mm^2 of tissue compared to 17.7 (S.E of the mean 0.12) for IEs. Also, IE GUS foci were on average 2.7 x larger than those from microbulbs (average from 1500 foci). The higher numbers and greater size of GUS foci in IEs compared to microbulbs suggest that, on average, individual IE cells were more competent at accepting and expressing the *uidA* gene.

Cultivar and tissue age also had significant effects on the number of foci generated. Table 1 (experiment 5) shows that microbulbs cultured for one week generated more foci than microbulbs cultured for 2-10 weeks prior to bombardment (the increase in GUS transient activity between 4 and 8 weeks may have been caused by the sub-culture of embryos to fresh medium three days prior to the eight week bombardment).

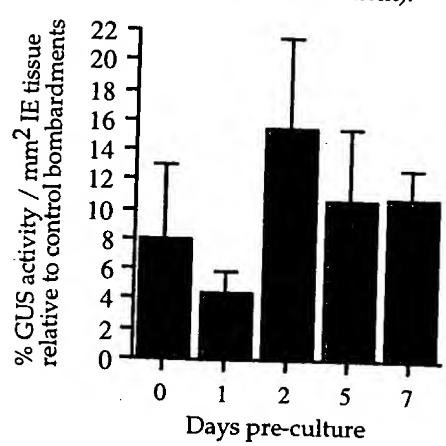

Fig. 2. The effect of pre-culture on transient GUS activity. IE material bombarded with pAHC25. Error bars represent the S.E. between three experiments.

For immature embryos, 2-5 day pre-culture was optimal (Fig. 2), as has been demonstrated previously

in maize IEs (Songstad et al. 1993). Other reports for barley IEs indicated that immediate bombardment was optimal (Wan and Lemaux 1994). In our system, the IEs were isolated from umbels which had spent 4 -10 days in transit in cool boxes. Metabolic stress during post harvest periods is known to rapidly alter gene expression (Davies and King 1993). Variation between cultivars existed and in the case of Sapporo Yellow, large variation was observed within the cultivar suggesting a strong genotype influence on *uidA* expression (Table 1, experiment 5).

Effect of promoter-uidA construct on bombardment

Six different promoter -*uidA* constructs were tested on IEs or microbulbs to determine which gave the highest level of *uidA* expression. The constructs pAHC25 (Christensen et al. 1992), pAoPR1 (Ozcan et al. 1993), pAct1-D (McElroy et al. 1990) and pEMU (Last et al. 1991) were chosen as previous work had demonstrated their suitablity in monocotyledonous systems. pART8 (Gleave 1992) and p35SGmtx (Detlef Becker unpublished), containing the CaMV35S promoter (Harpster et al. 1988) were chosen as the CaMV35S promoter drives high levels of expression in dicotyledonous systems (Wilmink and Dons 1993). Fig. 3 shows that transient GUS activity was 4-5 fold greater when construct pART8 was used. pAHC25 promoter activity was checked by bombardment into barley IEs and performed considerably better than pART8 in this tissue (data not shown). p35SGmtx performed less well than pART8 despite having the same promoter. p35SGmtx is a larger plasmid (14 Kb) and contains an intron within the *uidA* gene, either of which may have affected plasmid stability and subsequent gene expression in onion.

The consistently higher levels of GUS activity produced by the CaMV35S-*uidA* construct in pART8 was not initially anticipated as the other promoters were designed for expression in monocotyledonous species. The CaMV35S promoter has been shown to function in tulip and asparagus (Delbriel et al. 1993, Wilmink et al. 1992), which like onion are members of the super order Liliiflorae (Dahlgren et al. 1985). It may be that this monocotyledonous family behaves more like typical dicotyledonous species with respect to CaMV35S promoter function.

Agrobacterium-mediated transformation

Agrobacterium-mediated transformation produced foci over the entire surface of the embryos or microbulbs. These could not be readily quantified using image analysis; instead foci were counted under a binocular microscope. Also, transformation occurs within a cocultivation period of 5 days;.therefore transient activity, measured two days after this, reflected a two to seven day accumulation of GUS product, making comparison between the two systems difficult. It was noted that the tissues transformed using *Agrobacterium* containing the CaMV35S-tobacco mosaic virus leader - *uidA* gene construct within the T-DNA turned blue quickest when histochemically stained, compared to constructs introduced in bombardment experiments, indicating that this promoter-leader regulatory sequence may further enhance expression in onion tissues. Staining resulting in distinct foci was attributed to plant cell derived GUS activity and not from contaminating bacteria as the *uidA* gene used in the modified pGA643 lacks a bacterial ribosomal binding site and shows negligible expression in bacteria (Janssen and Gardener 1989). The number of transient GUS foci generated by inoculation with *Agrobacterium* was 1.6 and 4.9 foci per explant for microbulbs and IEs respectively. Approximately 3 fold more spots were generated on IEs than microbulbs. However, this was between 17 and 34 fold less than the number of foci generated by bombardment.

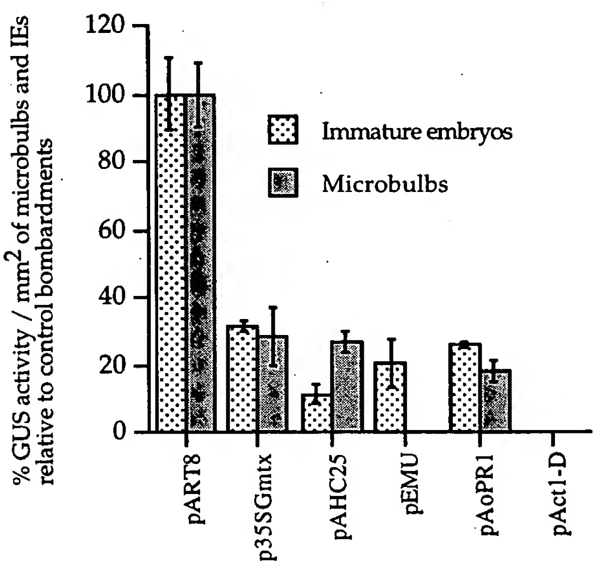

Fig. 3. The effect of different plasmids containing promoter - *uidA* constructs on GUS activity relative to control bombardments with pART8. Error bars represent the S.E. between three experiments.

Summary

In this study we have demonstrated that *uidA* can be delivered into onion tissues by *Agrobacterium* -mediated delivery or by microprojectile bombardment. Using transient expression analysis, we have optimized the physical parameters and shown that the CaMV35S promoter sequence drives high levels of expression in onion. The results of this study are now being used to develop constructs containing highly expressed selectable genes which will be used to produce transgenic onion plants.

Ackn wledgements

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(54) **Plant transformation method using agrobacterium species.**

(57) Plant cells are transformed by bombarding them with microparticles in a typical particle gun and then treating the bombarded cells with bacteria of the genus Agrobacterium which have been transformed to incorporate the DNA sequence of interest into their T-DNA. High frequencies of stable transformation are achieved.

EP 0 486 233 A2

T chnical Field

The present inv ntion relates t the use of Agrobacterium species for the transformation of plants.

Ba kground Art

Much research in plant molecular biology is now directed to the improvement of plant varieties via use of recombinant DNA techniques. Historically, plant breeders used classical genetic techniques to identify, preserve and crossbreed varietal lines having desirable traits. More recently, new plant varieties were induced by chemicals or by radiation treatment to mutate plant cells which were then regenerated using tissue culture techniques. These random and unpredictable approaches have obvious drawbacks. By the use of recombinant DNA technology, specific genes producing specific proteins, such as those imparting insect resistance, can be introduced into a plant to produce a desired variety with a particular trait.

Plants have been transformed using a variety of methods. A common method for transformation of dicotyledonous plants has been the use of disarmed Agrobacterium species, which are relatively benign natural pathogens of dicotyledonous plants. Agrobacteria infect plants and cause a callus of tumor tissue to grow in an undifferentiated way at the site of infection. The tumor inducing agent is the Ti plasmid, which functions by integrating some of its DNA into the genome of host plant cells. This plasmid is an ideal vector for transformation of plants. The portion of the Ti plasmid DNA that is transferred to host cell chromosomes during Agrobacterium infection is referred to as transforming ("T") DNA. See, for example, Watson JD, Tooze J, & Kurtz DT, Recombinant DNA: A Short Course, 169 (W.H. Freeman, 1983).

While early studies with Agrobacterium suggested that dicots were completely insensitive to this pathogen, those conclusions were based on lack of observable tumor formation in inoculated plants. More recently, it has been found that tumor formation in dicots is attributable to overproduction of auxins and cytokinins caused by the Ti plasmid, and therefore this symptom is not always a reliable indicator of transformation. More sensitive and more recent studies have shown production of opaline and nopaline, also attributed to the Ti plasmid, in Agrobacterium-inoculated monocots, and genetically engineered marker genes, such as GUS and NPTII, have been found in progeny of Agrobacterium-transformed corn plants. However, the successful and reliable use of this method still tends to be genotype specific both as to plants and Agrobacterium, as well as culture medium specific. Even under good conditions, the frequency of transformation is relatively low in some species.

In addition, Agrobacteria normally require a wound environment to induce the DNA transfer needed for transformation. For example, leaf punches and stem segments are commonly used because they present a cut and wounded surface to the bacteria that may contain cells capable of regenerating plants. There are times, however, when the intended target is an organized, multilayered tissue, such as a meristem, which is not readily accessible for Agrobacterium infection and transformation and is not easily wounded without damaging its organization and function. Even where leaf punches and stem segments are used, these only present a limited region, such as the perimeter of a leaf punch disk, which has been wounded. It would be desirable to use the entire surface of the disk as a potential transformation site.

Another method for transformation of plants has been bombardment of plant cells with dense microparticles carrying genetic material such as DNA sequences or plasmids. This method is less genotype specific, but frequencies of stable transformation are also low with this method. This is due in part to an absence of natural mechanisms to mediate integration of the introduced genetic material into the plant genome. In contrast, Agrobacterium species actively mediate those transformation events as a part of the natural process of infecting a plant cell. Thus, a continuing need exists for a method of transformation which reduces genotype specificity and enhances reliability, both in monocots and dicots.

Brief Description of the Drawings

Figures 1 through 4 are plasmid maps of the plasmids pPHI158, pPHI167, pPHI419 and pPHI413, respectively.

Disclosure of the Invention

This invention provides an improved transformation method in which plant tissues are first perforated with microprojectiles which d not carry g n tic material. It is now appreciated that th high v locity impact of d nse particl s n plant tissues will g n rate a wid array of microwounds, creating an environment which is particularly conducive to infecti n by Agrobact ria. In the n xt step, th tissu s are treated with an Agrobacterium species carrying the g n tic material of interest. The Agrobacterium is able to transfer genetic material perma-

n ntly into the g nome of target cells at frequ ncies substantially high r than by conv ntional Agrobacterium treatment. Whil excessive wounding of th targ t tissu is d trimental in conventional particle/ plasmid methods of transformation, such wounding is used to advantage in the present invention. Accordingly, th present inventi n provides a method for transformati n f cells of a plant, comprising th steps of (a) preparing bacteria of an Agrobact rium species, which bacteria have be n transformed to include in th ir T-DNA th g netic material to be inserted into the genome of the plant cells; (b) perforating a tissue from the plant by microprojectile bombardment; and (c) treating the perforated tissue with the transformed Agrobacteria; whereby the Agrobacteria incorporate the T-DNA, including the inserted genetic material, into the genome of the cells.

This method can be used to make permanently, heritably transformed plant cells which can be regenerated to whole, fertile plants. Of course, it will be appreciated that the foregoing method can also be used for transient transformations and assays in plant research.

The transformed plant cells produced by the foregoing method are suitable for regeneration by known techniques to produce whole, fertile plants which include in their nuclear genome the genetic material incorporated by the action of the Agrobacteria. Accordingly, this invention also provides a method of producing whole, fertile, transformed plants, comprising the steps of (a) culturing tissues of the species and genotype to be transformed; (b) preparing bacteria of an Agrobacterium species, which bacteria have been transformed to include in their T-DNA the genetic material to be inserted into the genome of the plant cells; (c) perforating the target tissue by microprojectile bombardment using microprojectiles which do not carry genetic material; (d) treating the perforated tissue with the transformed Agrobacteria, whereby the Agrobacteria incorporate the T-DNA, including the inserted genetic material into the genome of the cells to produce transformed cells; and (e) regenerating the transformed cells to produce whole plants.

In many instances it will be desirable to regenerate plants from cultures which consist entirely or essentially of transformed cells, so that plants which are not chimeric can be obtained. This can be accomplished by growing the bombarded and Agrobacterium-treated tissue prior to regeneration in a selection medium in which only transformed cells are viable. This can be done by including a selectable marker gene such as kanamycin or Basta resistance in the plasmid to be inserted in the cells, as illustrated in Figure 2. When the treated cells are grown in a medium containing the antibiotic or herbicide, the chemical will destroy non-transformed cells, and the surviving cultures will consist entirely of transformants, which can then be regenerated to produce plants which are not chimeric.

While not intending to be limited by theory, normal microparticle bombardment schemes require that individual or very small groups of particles enter the target cells in such a manner and location that the cells remain competent for division. In contrast, it is believed that Agrobacterium transformation occurs when the bacteria bind to the surface of a target cell. It is only the bacterial T-DNA that is "injected" into the cell, once the bacteria are induced by the wound environment to activate their virulence and transfer functions. Thus it will be appreciated that the objective of bombardment in the practice of this invention is to induce cell wounding and death to a certain extent, rather than to minimize wounding as is desirable with the conventional practice of bombarding with DNA-loaded particles. Once an area is damaged and releases the set of cell metabolites and wound exudates which Agrobacteria recognize, the remaining intact cells in the region of the wound are the transformation targets, rather than the cells which have been hit by particles. Accordingly, in the practice of this invention the particles need not and preferably do not carry genetic or other biological material.

Plants and Plant Cells

This method can be employed with any desired agronomic or horticultural species, including both monocots and dicots. As evidenced by the results achieved in sunflower, the higher transformation frequencies obtained with this invention can overcome in part the low frequencies of transformation associated with many difficult to tranform genotypes and species. Preferably, the monocot species will be selected from maize, sorghum, triticale, barley, oats, rye, wheat, onion and rice, and the dicot species will be selected from soybean and other beans; alfalfa; tobacco, brassicas such as rapeseed, broccoli and cauliflower; sunflower; cucurbits such as melons, cucumbers and squashes; and solanaceae such as potatoes, peppers and tomatoes. Tissues from flowers, including orchid, rose, carnation, petunia, zinnia, aster, lily, marigold, impatiens, African and common violet and pansy, anthurium, gladiolus, hyacinth, geranium, lavender, peony, tulip, poppy, chrysanthemum, daffodil, and begonia varieties, as well as other ornamentals, including without limitation taxus, juniper, rhododendron, philodendron, ficus, ivy, pothos, lilac, cactus, dizygotheca, euphorbia, fatsia, hedera, coleus, and other vari ti s, and herbs such as parsl y, sag , rosemary, thyme, basil, oregano, garlic, mint, f nnel, marjoram, coriand r, dill, and the lik can also be subjected to th methods of this inv ntion.

Tissues used can come from any desired plant part, including roots, anthers, stems, cotyled ns, hypocotyls and flow rs. Preferred tissues include meristem xplants, whole leaf xplants, partial leaf cuttings, leaf punch

disks and immature embryos. An especially preferred tissu is a split meristem explant. This latter tissue has been described in th literature by B. Schramm ijer t al., "Meristem Transformation of Sunflower via Agrobacterium," Plant Cell Reports 9: 55-60 (1990).

Agrobact rium Species

Species of Agrobacterium which can be used in plant transformation include Agrobacterium tumefaciens and Agrobacterium rhizogenies. Preferred is an Agrobacterium tumefaciens strain of the nopaline, binary type. Especially preferred is the publicly available Agrobacterium tumefaciens strain EHA101. This strain contains a C58 bacterial chromosome and a disarmed derivative of the Ti plasmid referred to in the literature as TiBO542. [See, e.g., Hood EE, Helmer GL, Fraley RT & Chilton M-D, "The Hypervirulence of Agrobacterium tumefaciens A281 is Encoded in a Region of TiBO542 Outside of T-DNA." J. Bacteriology 168:1291-1301 (1986)].

While selection and transformation of Agrobacterium does not per se form a critical part of this invention, in a preferred embodiment strain EHA101 is transformed with plasmids pPHI158 and pPHI167 as shown in Figures 1 and 2, using freeze-thaw transformation. pPHI158 (Figure 1) is constructed by the insertion of linearized, EcoR1 digested plasmid pPHI419 (Figure 3) carrying the plant-expressible marker NPTII near the right border of the 11.6 kb binary pPHI6. pPHI6 also contains the RK2 origin of replication and an ampicillin resistance marker. pPHI167 is constructed in an identical manner except that the linearized EcoR1 fragment of pPHI413 (Figure 4) carrying the GUS gene is inserted into pPHI6. This is referred to in the literature as a binary vector system. [See, e.g., Hoekema A, Hirsch PR, Hooykaas PJJ & Schilperoort RA, "A Binary Plant Vector Strategy Based on Separation of Vir- and T-Regions of the A. tumefaciens Ti Plasmid." Nature 303: 179-180 (1983).]

The bacteria are preferably grown in YEP medium supplemented with 50 μg/mL kanamycin and 100 μg/mL carbenicillin to an OD_{600} of 0.5-1.0. For inoculation of plant tissues the bacteria are preferably transferred to inoculation medium. Compositions of various media are as follows:

AB
- 3 g/L K_2HPO_4
- 1 g/l NaH_2PO_4
- 1 g/l NH_4Cl
- 0.3 g/L $MgSO_4 \cdot 7H_2O$
- 0.15 g/L KCl
- 0.01 g/L $CaCl_2$
- 2.5 mg/l $FeSO_4 \cdot 7H_2O$

YEP
- 10 g/L Yeast Extract
- 10 g/L BactoPeptone
- 5 g/L NaCl

LB
- 5 g/L yeast extract
- 10 g/L Bactopeptone
- 10 g/L NaCl

all above at pH 7.0

Inoculation Buffer
- 12.5 mM MES at pH 5.7
- 1 g/L NH4Cl
- 0.3 g/L MgSO4

Induction Buffer
- $\frac{1}{2}$-strength AB medium
- 3% sucrose
- 20 mM MES pH 5.5
- 200 μM acetosyringone

Example 1

Sunflower transformation

A g n ral method for transformation of sunflow r merist m tissues is practiced as f llows.

Shelled unflower seed are surface sterilized with dilute hypochl rite solution in th usual manner and imbibed overnight (18 hours) in th dark at 26°C on moist filter paper to initiate germination. Th following morn-

ing, th cotyled ns and th emerging root radical are removed and th explant containing th meristem is cultured overnight on medium 374B-GA, which contains Murashige & Skoog minerals, Sh pard vitamins, 3% sucrose, 0.8% agar (Phytagar) and th hormones BAP (0.5 mg/L), IAA (0.25 mg/L) and GA (0.1 mg/L) at a pH of 5.6. 24 hours later th primary leaves are removed, xposing th apical meristem. Th meristems are arranged in a 2 cm circl in the center of a petri plate containing a stiff water agar to hold th meristems upright for bombardment purposes. The meristems are bombarded twice in a microparticle bombardment apparatus of the general construction described by Sanford et al. in their European Patent Application, Publication Number 331,885, claiming priority of U.S. Patent Application Serial No. 161,807, filed February 29, 1988, the disclosures of which are hereby incorporated herein by reference. Nitric acid-washed tungsten particles having a mean diameter of 1.8μm suspended in TE buffer are used, and the explants are positioned 10cm below the stopping plate.

Following the bombardments a small droplet of log phase Agrobacteria (containing the desired modified Ti plasmid) in inoculation buffer at a concentration of OD=2.0 at 600nm is applied to each meristem. The cultures are incubated on 374B-GA for 3 days and then transferred to 374 medium (374B-GA without hormones but containing 250 μg/mL cefotaxime to inhibit bacterial growth). The meristem plants emerge in a few days and are allowed to develop for about 2 weeks at 26°C with a 16 hour day. At that point plants can be harvested to measure the level of transformation that has occurred. This can be done, for example, 1) based on the number of stained leaf sectors observed using GUS histochemical staining when the GUS gene is used (submerging the tissue overnight in x-gluc), or 2) by observing the green regions under kanamycin selection when the plasmid transferred by the Agrobacterium contains the NPTII gene or under Basta selection using the BAR gene.

Example 2

Tobacco Leaf Transformation

Tobacco leaves were treated in the same general manner as described in Example 1. The culture medium comprised MS minerals and vitamins, 4% sucrose and 1.5% Gelrite at a pH of 5.8. 10 to 14 days post germination, the first true leaves were harvested and cultured adaxial side up for 24 hours on filter paper moistened with medium comprising MS minerals, B-5 vitamins, 4 mg/L pCPA, 3% sucrose and 0.15 M sorbitol at a pH of 5.8. The leaves were dipped for 10 minutes in an Agrobacterium tumefaciens EHA101/pPHI158 or EHA-101/pPHI167 suspension after bombardment and then returned to the culture medium, and 3 days later were transferred to 526 medium, comprising MS mineral elements, B-5 vitamins, 0.5 mg/L BAP, 2.0 mg/L NAA, 3% sucrose, and 0.8% Phytagar at a pH of 5.7, supplemented with 100 μg/Ml kanamycin sulfate and 250 μg/mL cefotaxime. The NPTII gene was used as a selectable marker and selection was done in a kanamycin-containing medium. Additional treatment groups included three levels of controls: Group II used bombardment followed by the same Agrobacteria as the test group containing same plasmid but without the NPTII gene (i.e., no selectable marker); Group III used the test Agrobacteria containing the test plasmid but without bombardment; and Group IV used standard particle/plasmid bombardment using the NPTII gene (i.e., no Agrobacteria) (Group IV). The combination of particle bombardment, Agrobacterium cultivations and kanamycin selection pressure did not prevent leaves from forming callus and regenerating plants. Transformation was identified by counting colonies surviving on the medium. Results were as follows:

Treatment group	# of Explants	Avg # of Colonies
I	41	15.8
II	68	0
III	64	Only at cut point
IV	66	0.77

From these results there appears to be about a 20-fold increase in the frequency of transformation using the method of this invention, compared to standard particle/plasmid methods.

Example III

Sunflower meristems were transf rmed in the g neral mann r of Example I. Meristem diameter increased from 50 μm to ov r 200 μm in 2 days in cultur prior to bombardm nt. Plants were easily recov red from meristems after treatm nt. Transf rmation was valuated by counting plants with GUS-positive sectors and comparisons were made using treatment groups divided as in groups I, III and IV of Example II. Results, expressed

as th percentag of transformants identified among reg n rated plants, were as follows:

Treatment group	% transformants
I	14.4
III	0
IV	0.1

From these results there appears to be about a 140-fold increase in the frequency of transformation using the method of this invention, compared to standard particle/plasmid methods.

Example IV

A more extensive experiment along the lines of Examples 1 and 2 were conducted using Xanthi (tobacco) leaves. Treatment groups were as follows:

Group	Wounding	Dipped	Bacteria	Plasmid
1	Particles[1]	No	None	None
2	Splitting	No	None	None
3	Particles[1]	Yes	EHA101	pPHI167
4	Particles[1]	Yes	None	pPHI419
5	None	Only	EHA101	pPHI158
6	Particles[1]	No	None	pPHI419
7	Splitting	Yes	EHA101	pPHI158
8	Particles[1]	Yes	EHA101	pPHI158

[1] Two bombardments

The selection medium included kanamycin, so that only cells transformed to contain the NPTII gene (pPHI158 or pPHI419)) were expected to survive. Cells transformed with pPHI167 (GUS gene) could have been identified by blue staining, but would not yield viable colonies in this experiment. Results were as follows:

Group	# Explants	Mean # Colonies	Colonies/Leaf
1	31	0	0
2	40	0	0
3	34	0	0
4	55	0	0
5	64	colony development at excision point only	
6	48	0.15±0.08	0.36
7	37	2.5±0.45	4.0
8	51	14.35±1.9	36.5

The first five groups were controls: Group 1 to evaluate the effect of bombardment only on colony development; Group 2 to evaluate the effect of wounding using a scalpel cut to split the leaf; Group 3 to evaluate particle wounding as in Group 1 but with functional bacteria that contained GUS only (no selectable marker) to establish a bas lin ; and Group 4 to valuate whether the Agrobact ria wer needed if microparticle wounding is us d. None of these treatm nts were xpected t produce viabl colonies n s lecti n medium, and all of these dev l ped 0 colonies on sel ction medium. Group 5 was a control to confirm th need for tissue wounding, and col nies developed only at th excision point as expected.

Group 6 was a positive control using normal particl gun m thods, and Group 7 was a positiv control using wounding by a simple scalpel cut to split the leaf. Group 8 used the m thod of this invention. Th method of this invention showed approximately a 100-fold improvem nt in frequency of transformation compared to a conv ntional microparticl bombardm nt meth d and a 9-fold improvement in comparison to a conv ntional Agr bacterium transformation method which used a split leaf specim n.

Example V

The methods of this invention were compared to the method in which tissues are bombarded with microparticles to which transformed Agrobacteria have been applied, as described in my copending application Serial No. , filed November 14, 1990. Results were as follows:

Bombard	Dia.	Material	Conc.	Meristems	Sectors	%
w/bact.	~1.3μm	gold	Dried	121	4	3.3
			in YEP	125	3	2.4
2x pre	1.8μm	tungsten	OD=2	106	8	7.5
				138	21	15.2
4x pre	1.8μm	tungsten	OD=2	54	8	14.8
6x pre	1.8μm	tungsten	OD=2	83	9	10.8
2x pre	1.3μm	tungsten	OD=2	73	15	20.3
2x pre	1.8μm	tungsten	OD=2	80	14	17.5
2x pre	2.4μm	tungsten	OD=2	70	8	11.4
2x pre	1.8μm	tungsten	OD=2	39	7	17.9
2x pre	1.8μm	tungsten	OD=4	75	7	9.3
2x pre	1.8μm	tungsten	OD=6	74	7	9.5

From this it was concluded that although bombarding tissues with Agrobacteria dried onto particles is an effective method of transformation using Agrobacteria, it is not as effective as bombarding first and then applying Agrobacteria as a droplet of suspension to the wound site.

Claims

1. A method for transformation of cells of a plant by insertion of genetic material into the genome of the cells, comprising the steps of
   (a) preparing bacteria of an Agrobacterium species, which bacteria have been transformed to include in their T-DNA the genetic material to be inserted into the genome of the cells;
   (b) perforating a tissue from the plant by microprojectile bombardment; and
   (c) treating the perforated tissue with the transformed Agrobacteria;
   whereby the Agrobacteria incorporate the T-DNA including the inserted genetic material into the genome of the cells.

2. A method according to Claim 1 wherein the tissue is a meristem explant.

3. A method according to Claim 1 wherein the tissu is a member selected from the group consisting of wh le leaf xplants, partial leaf cuttings, and leaf punch disks.

4. A method according to Claim 1 wherein th tissu is immature mbry s.

5. A method according to any ne of the preceding claims wh rein th plant is a monocot selected from th group consisting of maize, sorghum, tritical , barley, oats, ry , wheat, oni ns and rice.

6. A method according to any one of claims 1 to 4 wherein the plant is a dicot selected from th group consisting of soybean, alfalfa, tobacco, brassicas, sunfl wer, cucurbits, potatoes, p ppers and tomatoes.

7. A method of producing whole, fertile, plants, the cells of which have been transformed by insertion of genetic material into their genome, comprising the steps of
(a) preparing bacteria of an Agrobacterium species, which bacteria have been transformed to insert in their T-DNA the genetic material to be inserted into the genome of the plant cells;
(b) perforating by microprojectile bombardment a tissue from a plant of the species and genotype to be transformed;
(c) treating the perforated tissue with the transformed Agrobacteria, whereby the Agrobacteria incorporate genetic material comprising the inserted genetic material into the genome of the cells to produce transformed cells; and
(e) regenerating the transformed cells to produce whole plants.

8. A method according to any one of claims 1 to 7, further comprising the step of growing the bombarded, Agrobacterium-treated tissue in a selection medium in which only transformed cells are viable, prior to regeneration.

9. A method according to claim 7 wherein the tissue is as defined in any one of claims 2 to 4.

10. A method according to claim 7 wherein the plant is as defined in claim 5 or 6.

Figure 1

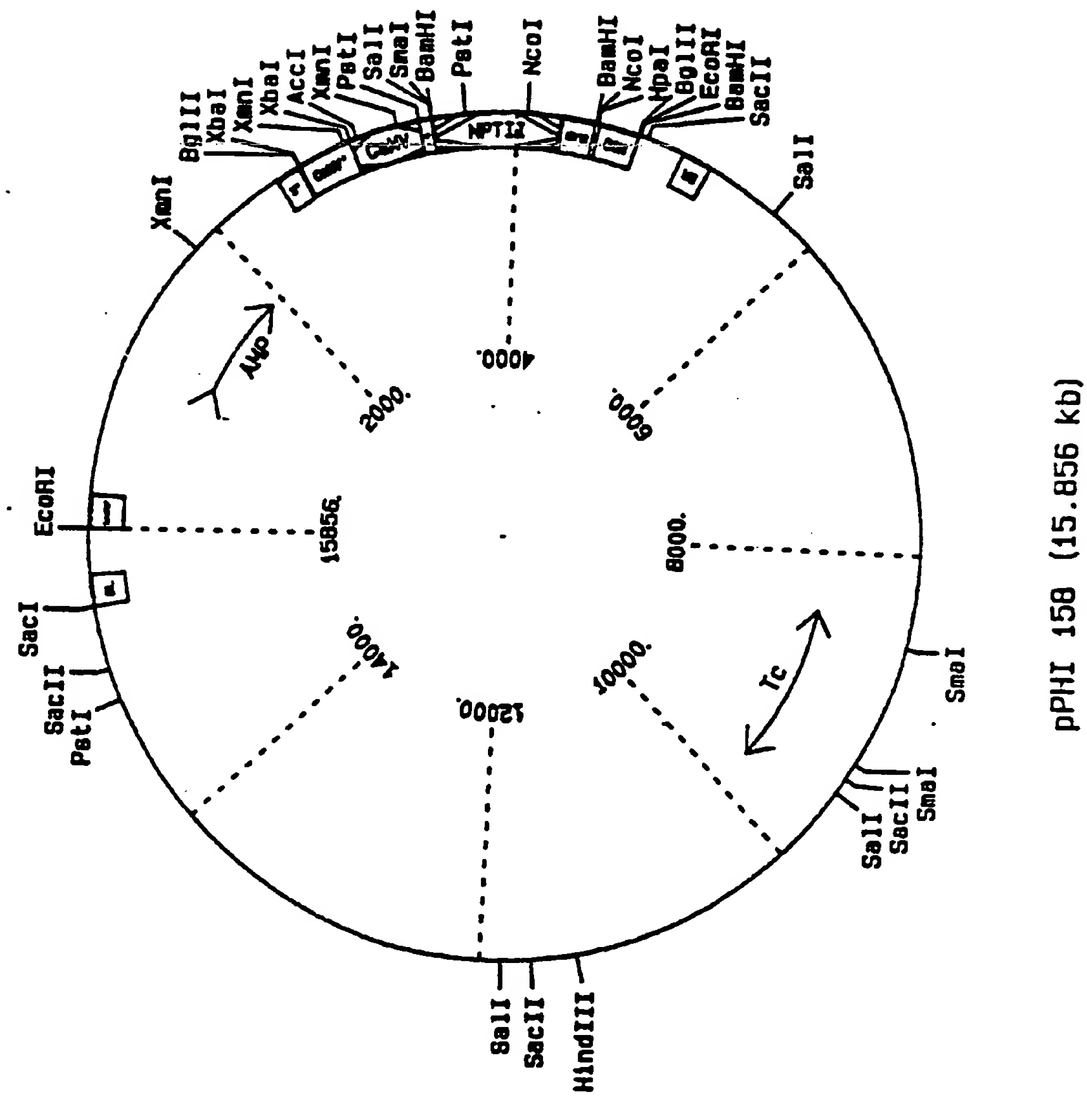
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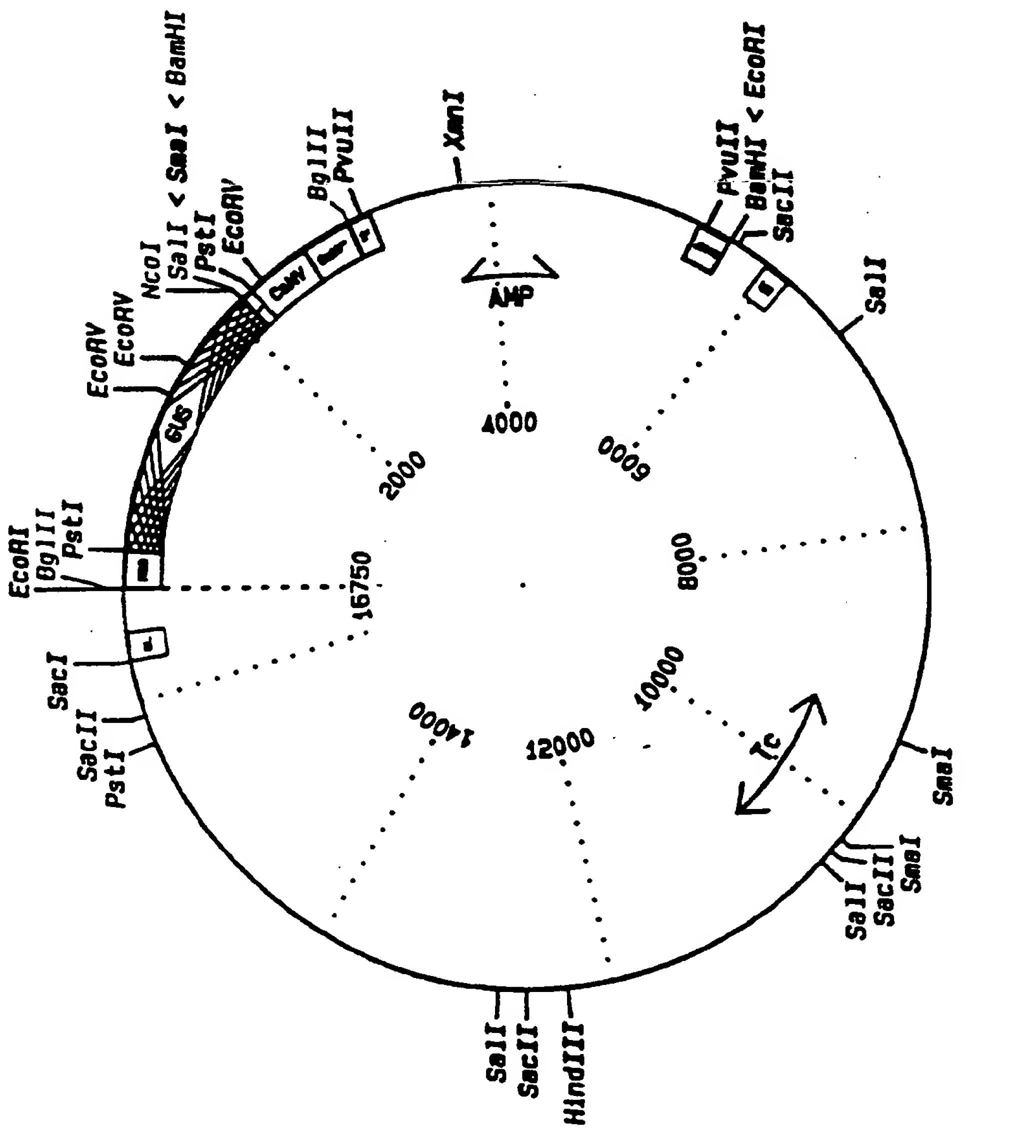
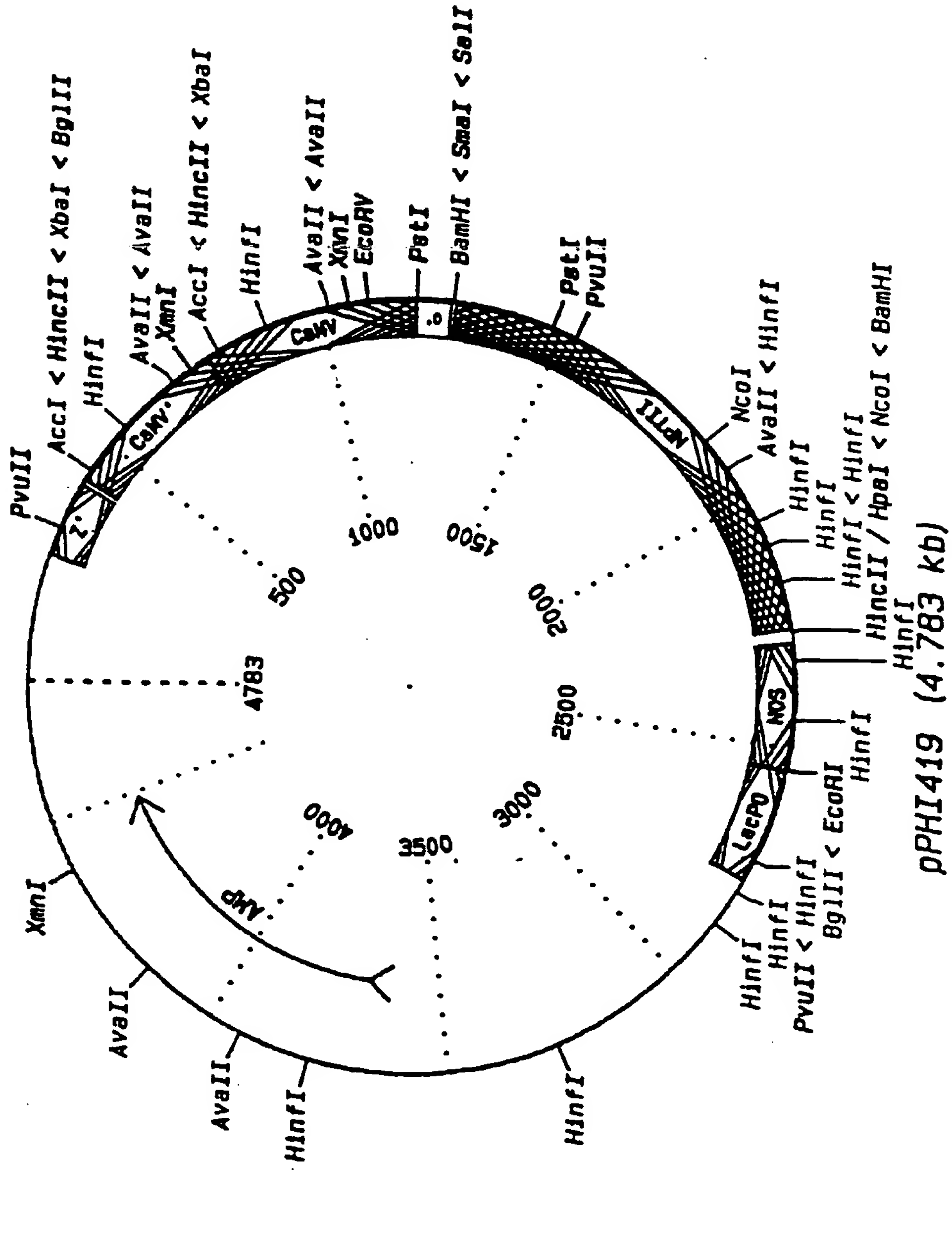
Figure 2



pPHI 167 (16.75 kb)

Figure 3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AccI < HincII < XbaI
HinfI
AvaII < AvaII
XmnI
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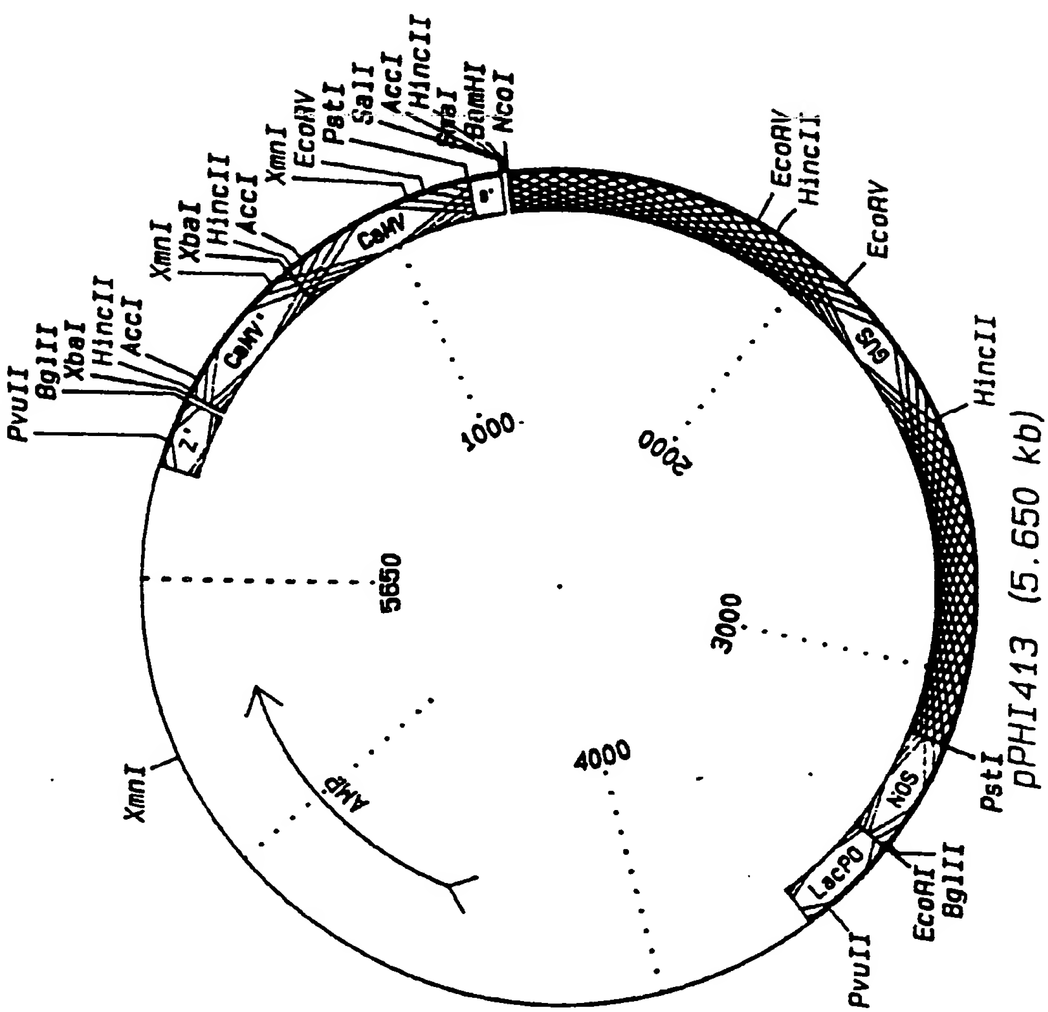
HinfI
HinfI
HinfI
HinfI
AvaII
AvaII
XmnI
CaMV
NPTII
NOS
LacPO
Amp
500
1000
1500
2000
2500
3000
3500
4000
4783

Figure 4


pPHI413 (5.650 kb)
PvuII
BglII
XbaI
HincII
AccI
XmnI
XbaI
HincII
AccI
XmnI
EcoRV
PstI
SalI
AccI
HincII
SmaI
BamHI
NcoI
EcoRV
HincII
EcoRV
HincII
PstI
EcoRI
BglII
PvuII
XmnI
2'
CaMV
CaMV
GUS
NOS
LacPO
AMP
1000
2000
3000
4000
5650